Investigating the Effect of Microgravity on Adaptive Immune Response to Viral Infection

Rebecca Richardson

Arizona State University

MIC 401

Reader: Dr. Joseph Blattman

Abstract

Microgravity conditions associated with space flight have been shown to cause immune deficiencies in a number of ways, such as decreasing hematopoietic differentiation, cytokine production, and lymphocyte proliferation[1]. Space flight has also been shown to cause changes in DNA fragmentation and changes in lymphoid organ size in mice models[2]. Research on immunology in spaceflight became a prevalent topic when NASA published results of a significantly high re-emergence of latent viruses in astronauts returning from space missions, with additional studies showing decreases in cytokine activation and virus-specific T cells[3, 4]. The evidence for immune system impairment in microgravity conditions is particularly concerning given the plans for longer duration spaceflights for humans. Further research needs to be conducted to better understand the effect of microgravity on immune function. This proposed experiment will investigate the effect of microgravity on adaptive immune response to viral infection by inoculating mice with a virus and studying the immune responses of mice in simulated microgravity conditions. It is hypothesized that the mice in the simulated microgravity condition will show a lower antibody response to LCMV than the control group. Data will be obtained through the use of plaque assays, intracellular cytokine staining, tetramer staining, and ELISAs. Based on previous findings, is anticipated that the hypothesis will be supported; however, aberrant results would still provide insight to the role of microgravity on immune function and response.

Introduction

The first landing on the moon was in 1969, and NASA proposes to have humans land on Mars by 2030. Outer space is an exciting scientific frontier to explore, but the factors associated with spaceflights of any duration can lead to significant impairment of health. Unsurprisingly, leaving the earth's orbit brings about a number of unfamiliar circumstances to the individuals who venture out of the atmosphere. The rocket's takeoff results in a massive exposure to G forces, and the missions themselves tend to result in severe sleep deprivation, nutritional deficiencies, and anxiety or depression[1]. In addition, research shows that the immune system suffers from the microgravity conditions experienced.

Microgravity refers to conditions where the force of gravity is so low that feelings of weightlessness occur. Gravity is generally measured by the speed at which an object would fall in free fall. On Earth, gravity is $9.807 m/s^2$. This value significantly decreases once Earth's atmosphere is exited—on Mars the force of gravity is $3.711 m/s^2$, and on the moon, gravity is $1.622 m/s^2$ (5). These significant decreases in gravity lead to observable impairment in immune function for a number of species. *Drosophila melanogaster*, the common fruit fly, showed a complete inability to activate Toll-mediated responses to fungal infections. The stress of microgravity on the body resulted in a faulty transcriptional response. Interestingly, hypergravity conditions, where the force of gravity was higher than experienced on earth, actually improved the signaling strength of Toll-responses, resulting in a faster clearing of the fungal infection[6].

In mice models, a number of immune changes have been observed. Lymphocyte proliferation is decreased due to a downregulation of T-cell activation markers CD25, CD69, and CD71. Decreased lymphocyte function and proliferation, especially for CD4 T-cells, occurred in a time-dependent manner with exposure to microgravity conditions[7]. Microgravity causes a significant unloading of mammalian tissues, which decreases tissue growth and regeneration. As a result, mice showed decreased hematopoiesis due to down-regulation of gene-expression markers for early mesenchymal and hematopoietic differentiation by at least two-fold. Cultures of bone marrow cells after microgravity conditions showed increase of mesenchymal differentiation to mineralized bone nodules, whereas hemotopoietic differentiation primarily resulted in osteoclasts. This indicates an increase of undifferentiated progenitor cells after microgravity[8]. Mice also show a decrease in lymphoid organ size relative to their body mass, with a 13-day spaceflight showing a significant decrease in spleen size. Thymus size was not significantly affected, but an increase in DNA fragmentation in the thymus was observed. T-cell and cancer gene expression markers were highly altered, with 30 out of 84 T-cell genes altered, and 15 out of 84 cancer related genes altered[2]. This evidence suggests that there could be an increased risk of infection and cancer development associated with spaceflight. IFNγ production is of particular importance, as it is the first response to viral infections. Rodents showed a decrease in IFNγ production in response to T-cell mitogen ConA[9]. The decrease in IFNγ present in mice causes Swiss/Webster mice that are normally immune to the D variant of encephalomyocarditis to become unusually susceptible in microgravity conditions[10].

A further investigation of rodent models also indicated an overproduction of cytokines IL-6 and IL-10, and a decrease in TNFα in microgravity conditions[11]. Humans also show an impaired immune response during microgravity conditions experienced during spaceflight.

Astronauts show decreased functionality of their monocytes, with reduced ability to engulf *E. coli*, induce oxidative burst or degranulate. In addition, responses to gram-negative LPS endotoxins have decreased responsiveness[12]. These alterations in immunity are likely due to decreased expression of CD14 and increased expression of TLR4, due to LPS responsiveness depending on the association of the CD14-TLR4 myeloid differentiation protein 2 complex[1].

Cytotoxicity of non-MHC-restricted killer cells was found to be depressed in spaceflight as well, with a 40% decrease in lytic activity after landing[13]. Cytokine expression changes have also been observed in astronauts, suggesting further indication of decreased natural immune response. Astronauts returning from a 7-day spaceflight showed low NK cell activity and decreased IFN secretion, which would reduce the ability to respond to viral infections[14].

The study of microgravity conditions on immune function became an especially prevalent topic when NASA observed a higher re-emergence of latent viruses in astronauts on short-duration space flights of 12 to 16 days. Astronauts showed increased re-emergence of Epstein-Barr virus, cytomegalovirus, herpes simplex I, and varicella virus[3].

NASA conducted further studies to investigate the alarming immune deficiencies in astronauts with simulated microgravity experiments. Research participants were found to have a decrease in activation of CD8+, CD69+, and virus-specific T-cells for both CMV and EBV[4].

Research on immune function in microgravity conditions is highly relevant due to NASA's plans to send humans to Mars by mid 2030s—this mission would be a duration of a minimum of 520 days[15]. Additionally, bacteria actually survive better in microgravity conditions, leaving humans at an even greater risk for infections. Bacteria are found to proliferate faster, improve gene expression and pathogenesis, and require higher doses of antibiotics to kill. The increased radiation in space also subjects bacteria to higher levels of mutations, which could lead to increased virulence[1]. Spaceflight also includes inherent risks that could increase susceptibility to infections, such as the emotional stress due to confinement or fear of failure. Air, food, water, and waste are all recycled on board the space crafts, and the confinement of the passengers has been shown to increases in the transfer of microorganisms[16, 17]. The role of gravity on immune cells needs to be established before humans are sent on space missions lasting such an exorbitant amount of time, especially considering the evidence of decreased immune function in short-duration spaceflights. Further research could definitively establish a connection between mechanical unloading of tissues negatively impacting hematopoiesis, cytokine production, and adaptive and innate immune responses.

Despite the compelling research suggesting significant immune impairment in microgravity conditions, gaps in scientific knowledge exist on the adaptive immune response to specific viral infections in microgravity conditions. This research will investigate the hypothesis that adaptive immune responses will be deficient in microgravity conditions when exposed to viral infection. It is suspected that mice subjected to microgravity conditions will show a lower antibody response of IFNγ and TNF at 30 days than control groups.

Specific Aims

Aim 1: Eliminate possibility of extraneous factors associated with spaceflight besides microgravity leading to evidence of decreased immune function.

Studies on microgravity conditions can be simulated due to the excessive expense of sending research subjects and participants to space. Head-down bed rest (HDBR) has been established as a sufficient method of simulating microgravity conditions[3]. It involves being placed at a -6 degree angle with the head down, restrained at the chest and ankles (see Figure 1). This method can be used on a variety of experimental models, including mice.

Figure 1: Visual representation of head down bed rest conditions to simulate microgravity conditions.

This method of establishing microgravity conditions rather than sending the research subjects to space will ensure the results obtained are not due to other factors, such as increased levels of radiation, stress levels, confinement, or improved microorganism proliferation.

Aim 2: Observe if T-Cell functionality is impaired in microgravity conditions, and determine the effect of microgravity on adaptive immune responses to viral infection.

Lymphocytic choriomeningitis virus (LCMV) is a prominent model for investigating the immune system's response to viral infection[18]. LCMV's natural host is a mouse, but it can also infect a wide range of animals, including humans. The Armstrong strain of LCMV, which will be used for this experimental design, was first isolated from a monkey in the 1930s.

It is referred to as the neurotropic strain due to its high levels of replication in the viscera, which clonally exhausts T cells. This prevents T-cell dependent meningitis and encephalitis from occurring, and results in an acute infection[18]. LCMV is a Biohazard Safety Level 2 (BSL-2) pathogen.

The LCMV pathogen will be injected into research subjects, and their immune responses will be studied using plaque assays, intracellular cytokine staining, tetramer staining, flow cytometry, and ELISAs. Results will be compared to mice in a control setting.

Experimental Design:

A total of 30 BALB/c mice between 8 and 10 weeks of age will be obtained from Jackson Laboratories for this experiment. The mice will be kept following Arizona State University and IUPAC regulations. Mice will acclimate for a week in normal conditions (no microgravity simulation) in groups of five in HEPA-filter caged racks. Mice will be provided water ad libitum, and food in a quantity of one lab block per day distributed at the same time each evening. Biosafety Level 2 precautions and Class II biosafety cabinets will be used at all times.

After 1 week, 20 mice will be restrained in the HDBR position. Mice will be placed prone on the HDBR block at -6 degrees to allow for easier feeding. 10 mice will be placed prone on a flat surface to serve as a positive control.

Viral stocks will be prepared by performing a serial dilution of the LCMV stock solution from 10^{-3} to 10^{-9}.

Dilutions will be used to infect cell VERO cell monolayers and to determine viral titer[19]. Ten of the mice will be injected with $2x10^5$ PFU of LCMV intraperitoneally, following protocol from Guidotti et. al[20]. Ten mice in HDBR condition will not be infected with LCMV to serve as a negative control. See **Figure 2** for explanation of experimental conditions.

Experimental Group	LCMV Infection	No LCMV Infection	Type of Control
10 Mice HDBR	X		n/a
10 Mice HDBR		X	Negative
10 Mice No HDBR	X		Positive

Figure 2: Indicates experimental groups involved in research design. 10 mice in HDBR with LCMV exposure are subjected to both experimental variables. 10 mice in HDBR conditions with no LCMV infection serve as a negative control, as no evidence of immune cell deficiencies specific to LCMV should be observed. 10 mice with no HDBR and exposure to LCMV are a positive control group, and will be compared to the mice in HDBR to quantify deficiencies in immune response to viral infection.

At three, eight, and thirty days time post-infection, mice from each experimental group will have their blood or splenocytes analyzed. Experimental design has been adapted from the protocol established in the Experimental Immunology lab manual for the purposes of this research[19].

Three days post-infection, a sample of blood will be obtained from 5 mice from each experimental condition. The serum will be used to perform a plaque assay to determine if the LCMV virus is present in cells. The plaques will quantitate the amount of virus present in the cells.

Eight days post-infection, 5 mice from each experimental condition will be euthanized and sacrificed. A sample of their splenocytes will be obtained, and tetramer staining, intracellular cytokine staining, and flow cytometry will be used to analyze the percentage of virus specific T-cells and the number of CD8+ and CD4+ T cells producing IFNγ and TNF.

To quantify number of virus specific T-cells, $1x10^6$ splenocytes from each experimental condition will be centrifuged for 1 minute at 8000 rpm. The supernatant will be decanted, and cells will be suspended in an antibody stain in FACS buffer in the following dilutions: anti-CD8 (eflouro450): 1/20, anti-CD4 (Percp-Cy5.5): 1/20, GP33 Tetramer (FITC): 1/10, and Anti-CD44 (PE): 1/10. Cells will be incubated for 30 minutes in 4° C in the dark. Cells will be centrifuged for 1 minute at 8000 rpm again, washed with FACS buffer twice, and resuspended in 200μL PFA buffer.

To determine the number of CD8+ and CD4+ T cells producing IFNγ and TNF, $1x10^6$ cells from each experimental group will be pipetted separately into 2 wells of a 96-well plate (6 wells total). Peptide dilutions with viral peptide epitope and Brefeldin A will be prepared in 10% FCS-RPMI+golgiplug to a final concentration of 2μg/mL each.

50μL of peptide will be added to wells that contain infected splenocytes. 50μL of 10% FCS-RPMI+golgiplug will be added to three empty wells, and 50μL of PMA/lonomycin will be added to three empty wells. Plates will be incubated for 6 hours at 37°C. Plate will be centrifuged at 1300 rpm for 2.5 minutes. Supernatant will be decanted, and 100μL of cell surface stain in the following dilutions in FACS buffer will be added to each well: Anti-CD8 (eflouro450): 1/20, Anti-CD4 (Percp-Cy5.5): 1/20. Plate will incubate at room temperature for 15 minutes in the dark, and be centrifuged for 2.5 minutes at 1300 rpm. Plate will be washed twice with FACS buffer, and 100μL of Perm/Fix solution will be added to each well. Plate will incubate for 20 minutes in the dark at 4°C. Plate will be washed twice with Perm/Wash buffer. 100μL intracellular stain in Perm/Wash buffer will be prepared in the following dilutions: Anti-TNF (FITC): 1/20, Anti-IFNγ (PE): 1/20. Plate will be incubated at room temperature for 15 minutes, then washed twice with Perm/Wash buffer, and finally resuspended in 200μL FACS buffer. Samples for both tetramer staining and intracellular cytokine staining will be run on an LSR Fortessa Flow Cytometer, and analyzed using Flowing 2.5.1 software.

Thirty days post-infection, an ELISA will be used to calculate the concentration of cytokines IFNγ and TNF in each experimental condition. The remaining mice (5 from each experimental group) will be sacrificed, and their splenocytes will be sampled. Protocol from the Experimental Immunology lab manual will be followed in order to prepare ELISA plate, with the exception of LCMV-infected supernatant being used in place of Poly(I:C) stimulated cell supernatant.

Unstimulated cells will be obtained from the HDBR mice with no LCMV exposure. Absorbance of the ELISA plate will be determined at 450 nm using a microplate reader[19].

Anticipated Outcomes

Based on previous research on the effect of microgravity on immune responses, the anticipated result from this experiment is that the hypothesis will be supported, and mice in the HDBR condition infected with LCMV will show a lower antibody response at 30 days than mice infected with LCMV in the control group. This would imply that the microgravity conditions negatively impacted the mice's T-cell responses, and the results from previous studies are indeed a result of microgravity, rather than extraneous factors associated with spaceflight. Other results that could be obtained could be that the mice in the HDBR condition actually show a better immune response to viral infections than mice in the control group. This seems highly unlikely based on previous findings, but if this result was obtained, further research could investigate the use of HDBR as a supplement to treating viral infections. Another possible result could be that the HDBR infected mice show no significant differences in antibody response at 30 days. If this data is obtained, an assumption could be made that other factors besides microgravity conditions alter the immune system's ability to resist pathogens. One possibility could be that the literature showing decreased immune function during spaceflight is a result of genetic mutations occurring due to exposure to increased radiation than experienced on Earth.

Another possibility could be that the cramped and confined positions astronauts face on small space shuttles is decreasing their immune response due to increased microorganism transfer. High stress levels associated with space flight could also impair the immune system. It has already been established that an increase in the level of cortisol, a hormone released when humans are under psychological stress, is found to decrease immune function and ability for wounds to heal[21]. Furthermore, the ability for bacteria to proliferate better in space could be what puts astronauts at highest risk, rather than the decreased immune function. Other explanations for the hypothesis not being supported could be due to microgravity conditions only negatively impacting innate immunity or responses to bacterial pathogens. Most of the research established on microgravity's effect on the immune system involves innate responses to bacterial pathogens, and investigating the adaptive immune response to viral infection is a new scientific approach. Therefore, the results of the proposed experimental design would contribute new and pertinent information on the function of the adaptive immune system in microgravity conditions, especially in a relevant day and age where longer duration spaceflights are planned.

References

1) **Gueguinou N, Huin-Schohn C, Bascove M, Bueb J, Tschirhart E, Legrand-Frossie C, Frippiat J.** 2009. Could spaceflight-associated immune system weakening preclude the expansion of human presence beyond Earth's orbit? Journal of Leukocyte Biology. **85**: 1027-1038.

2) **Gridley DS, Mao XW, Stodiek LS, Ferguson VL, Bateman TA, Moldovan M, Cunning CE, Jones TA, Slater JM, Pecaut MJ**. 19 September 2013. Changes in mouse thymus and spleen after return from the STS-135 mission in space. PLoSOne. doi:10.1371.

3) **Crucian BE, Stowe RP, Mehta SK, Yetman DL, Leal MJ, Quiriarte HD, Pierson DL, Sams CF.** May 2009. Immune Status, Latent Viral Reactivation, and Stress During Long-Duration Head-Down Bed Rest. Aviation Space and Environmental Medicine. **80**: 37-44.

4) **Kelson J, Bartels LE, Dige A, Hvas CL, Erings-Meuthen P, Boehme G, Thomsen MK, Fenger-Gron M, Dahlerup JF.** August 2012. 21 Days head-down bed rest induces weakening of cell-mediated immunity—Some spaceflight findings confirmed in ground based analog. Cytokine. **52:** 403-409.

5) **Minetti A.** 2001. Biomechanics: walking on other planets. Nature. **409**: 467-469.

6) **Taylor K, Kleinhesselink K, George MD, Morgan R, Smallwood T, Hammonds AS, Fuller PM, Saelao P, Alley J, Gibbs AG, Hoshizaki DK, VonKalm L, Fuller CA, Beckinham KM, Kimbrell DA**. January 2014. Toll mediated infection response is altered by gravity and spaceflight in Drosophila. PLoS One. doi: 10.1371

7) **Luo H, Wang C, Feng M, Zhao Y.** 2013. Microgravity inhibits resting T cell immunity in an exposure time-dependent manner. Int J Med Sci. **11**: 87-96.

8) **Blaber EA, Dvorochkin N, Torres ML, Yousuf R, Burns BP, Glubus RK, Almeida EA.** 2014. Mechanical unloading of bone in microgravity reduces mesenchymal and hematopoietic stem cell-mediated tissue regeneration. Stem Cell Res. **2:** 181-201.

9) **Gould CL, Lyte M, Williams J, Mandel AD, Sonnenfeld G**. 1987. Inhibited interferon-γ but normal interleukin-3 production from rats flown on the space shuttle. Aviat. Space Environ. Med. 58,983-986.

10) **Gueguinou N, Huin-Schohn C, Bascove M, Bueb J, Tschirhart E, Legrand-Frossi C, Frippiat J.** November 2009. Could spaceflight-associated immune system weakening preclude the expansion of human presence beyond Earth's orbit? Journal of Leukocyte Biology. **86:** 1027-1038.

11) **Baqai FP, Gridley DS, Slater JM, Luo-Owen X, Stodieck LS, Ferguson VL, SK, Pecaut MJ.** 2009. Effects of spaceflight on innate immune function and antioxidant gene expression. J Appl Physiol **106:**1935-1942.

12) **Rykova MP, Antropova EN, Larina IM, Morukov BV.** 2008. Humoral and cellular immunity in cosmonauts after the ISS missions. Acta Astraunautica. **63:** 697-70.

13) **Mehta SK, Kaur I, Grimm EA, Smid C, Feeback D, Pierson D**. 2001. Decreased non-MHC-Restricted killer cell cytotoxicity after spaceflight. Journal of Applied Physiology. **91:**1814-8.

14) **Talas M, Batkai L, Stoger I, Nagy K, Hiros L, Konstantinova I, Rykova M, Mozgovaya I, Guseva O, Kozharinov V.** 1984. Results of space experiment program "Interferon". Acta Astronaut. **11:** 379-386.

15) NASA. 2014. NASA's Journey to Mars. www.nasa.gov/content/nasas-journey-to-mars.

16) **Pierson DL, Chidambaram M, Heath JD, Mallary L, Mishra SK, Sharma B, Weinstock GM.** 1996. Epidemiology of *Staphylococcus aureus*during space flight *FEMS.* Immunol. Med. Microbiol. **16:** 273-281.

17) Pierson DL, Mehta SK, Magee BB, Mishra SK. 1995. Person-to-person transfer of *Candida albicans* in the spacecraft environment. J Med Vet Mycol. **33:** 145-150.

18) **Welsh R, Seedhorn M.** 2011. LCMV: Propagation, quantitation, and storage. Curr Protoc Micrbiol. Doi: 10.1002/9780471729259.

19) **Blattman J, McAffe M, Schoettle L.** 2013. Experimental Immunology.

20) **Guidotti L, Borrow P, Brown A, McClary H, Koch R, Chisari F.** 1999. Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte. J Exp Med. **10:** 1955-1564.

21) **Lucas VS.** 2011. Psychological stress and wound healing in humans: what we know. Wounds. **4**: 76-83.